

Cloning and expression of human neutrophil lipocalin cDNA derived from bone marrow and ovarian cancer cells

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Abstract Human neutrophil lipocalin (HNL) cDNA was amplified by PCR technology in combination with deoxyinosine containing oligonucleotides for cloning, sequencing and production of the recombinant protein in *E. coli*. The primers were targeted to the corresponding DNA backtranslate of the mature protein resulting in a PCR amplified 534 bp cDNA from different reverse transcripts of ovarian cancer cell line and bone marrow cell mRNAs. Sequence analysis revealed that two different cDNAs from ovarian cancer and bone marrow cells could be obtained. Cloning and expression of HNL cDNAs were performed in *E. coli* strain HMS 174 [DE3] using the pET system yielding in two recombinant proteins with a molecular weight of 21 kDa which is consistent with an 178 amino acid residues containing sequence of the mature HNL protein. N-terminal amino acid sequence analysis of the expression products showed an identical polypeptide sequence missing the *E. coli* processed starting methionine.

Key words: Lipocalin; Ovarian cancer; Inosine; PCR; Gelatinase

1. Introduction

HNL was first identified by Triebel et al. [1] as a 25 kDa gelatinase-associated glycosylated component of the 125 kDa gelatinase from human PMN leukocytes. Furthermore, the protein was localized in monomeric and dimeric forms in the specific granules of human neutrophils [2]. HNL belongs to the lipocalin family. It has strong homologies with the rat α_2 microglobulin-related protein [3] and the mouse oncogene protein 24p3 [4]. Most lipocalins are carriers of small hydrophobic substances through the hydrophilic medium of the body [5]. HNL has been shown to bind chemoattractants of the type of *N*-formyl peptides [6]. But its physiological role is still not clear. Although the amino acid sequence similarity among the members of the lipocalin family is low, there are three conserved regions containing the invariant residue tryptophan in one motif [7]. Crystallographic studies of several lipocalins revealed that they exhibit also a high similarity in the folding pattern with an eight-stranded antiparallel β -barrel fold into an orthogonal calyx [8]. Chan et al. [9] have shown that lipocalin genes are part of conserved synteny at HSA9q.

Here we report on both the PCR amplification using oligonucleotides containing deoxyinosine and on the sequencing of the

cDNA of the HNL from the reverse transcripts of ovarian cancer cell line and bone marrow cell mRNA. The obtained two different cDNAs could be cloned in *E. coli* and the rHNL could be expressed as a 21 kDa protein.

2. Materials and methods

Human bone marrow cells were a gift from Dr. F. Lindemann (Krankenanstalten Bethel, Bielefeld, Germany), human ovarian cancer cell lines OC 1, OC 2, OC 6 and OVCAR 3 were from Dr. O. Wilhelm (Frauenklinik der TU München, Klinikum r. d. Isar, Germany), *E. coli* strain HMS 174 [DE3] and plasmid vector pET 11a were from Novagen (Madison, USA), restriction enzymes were from Biolabs (Schwalbach, Germany).

2.1. RNA preparation and reverse transcription

Total RNA from 1×10^7 bone marrow cells and the ovarian cancer cell lines was prepared by the guanidinium isothiocyanate extraction procedure followed by ultracentrifugation through a cesium chloride gradient according to Chirgwin et al. [10]. First strand cDNA synthesis from 5 μ g of total RNA was performed with Superscript (Lifetechnologies, Inc.) by the manufacturer's protocol.

2.2. Oligonucleotide synthesis

Oligonucleotides were synthesized by Genosys Biotechnologies, Inc. according to the N-terminal and C-terminal amino acid sequences of the mature HNL protein. Deoxyinosine was introduced at the ambiguous codon positions in cases where more than two alternate deoxynucleotides would have been possible leading to following HNL-primers: 5'-CAGGACTCIACITCIGACCTIATCCCIGCCCC-3' and 5'-CCGTCIATGCAT TGATCIATIGGIACIGGGAA-3'.

2.3. PCR amplification and cloning of HNL

50 ng of first strand cDNA were used as template to amplify the HNL gene by PCR technology. Each reaction consisted of 100 pmol of primers, 0.2 mM dNTP, 1.5 mM $MgCl_2$ and 1 U of Taq DNA polymerase (Lifetechnologies, Inc.) in 50 μ l of reaction buffer recommended by the manufacturer. Two combined amplification cycles with 40 s at 95°C, 45 s 40°C, 90 s 72°C (5 cycles), 40 s 95°C, 60 s 50°C, 40 s +1 s/cycle 72°C (33 cycles) were carried out on a MiniCycler (MJ Research, Inc.).

2.4. Cloning and sequencing of the PCR amplification products

PCR fragments were electrophoresed in agarose gels and purified by phenol extractions and ethanol precipitation before ligating the PCR product into the TA cloning vector pCR II (Invitrogen Corp., San Diego, USA) using the manufacturer's protocol. This system takes advantage of the nontemplate dependent activity of thermostable polymerases used in PCR that add single deoxyadenosines to the 3'-end of all duplex molecules provided by PCR. Sequencing of the HNL cDNA in the plasmid pCR II was performed according to the dideoxy termination method [11].

2.5. Southern blot analysis

HNL cDNA containing plasmids pCR II and pET 11a were separately digested with *EcoRI*, *NdeI* and *BamHI* followed by agarose gelelectrophoresis, capillary transfer of restriction fragments on nylon membrane

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; OC, ovarian cancer; PMN, polymorphonuclear; TIMP-1, tissue inhibitor of metalloproteinases.

and hybridization using standard methods [12]. 'HNL PAT': 5'-CAA-GAG CTACAATGTCACCTCCGTCCTG-3' was labelled with digoxigenine (MWG-Biotech, Germany) and used as a probe in Southern blot hybridization.

2.6. Bacterial expression

For HNL expression the cDNA was selectively amplified with two oligonucleotide primers: 'HNLF' 5'-ATTCATATGCAGGACTCGA-CGTCGGAC-3' and 'HNLR' 5'-CAGGGATCC CTAGCCGTCGATGCATTGATCGA-3' using PCR II-HNL cDNA as template. 'HNLF' incorporates sequences for a unique *Nde*I site and an initiating methionine. 'HNLR' introduces sequences for a stop codon and a unique *Bam*HI site. Ligation into the plasmid pET 11a and expression in *E. coli* strain HMS 174 [DE3] followed as previously described [13].

2.7. SDS-PAGE and Western blot analysis

SDS-PAGE was performed by the method of Laemmli [14]. Western blots were performed as published by Bergmann et al. [15]. After blotting the proteins were further subjected to automated amino acid sequence determination in a Knauer sequencer by the method described in [16].

3. Results and discussion

Total HNL RNA was isolated from different ovarian cancer cell lines and bone marrow cells and used as templates for reverse transcription. Bone marrow cells were expected to be a good source for HNL mRNA as the protein was found to be expressed in human leukocytes [2]. Ovarian cancer cell cDNA seemed also to be a suitable source for HNL cDNA because the supernatant of these cells contain this protein (not published) as revealed by specific ELISA procedures [17].

Since only protein and no DNA information of HNL was available when we started these studies, the degeneracy of codons for amino acids had to be considered in the design of primers for the PCR technique. The use of selective oligonucleotides had to represent the potential nucleic acid sequences for each alternate codon. Oligonucleotide design which take ac-

count of this problems requires neutral bases like deoxyinosine [18]. Therefore HNL primers were designed by the DNA back-translate (Genetic Computer Group), containing five deoxyinosines each, according to the mature HNL amino acid sequence [19]. Fig. 1A shows the products of the PCR amplification reactions obtained with these oligonucleotides at the optimized low annealing temperature of 50°C. Variation of the annealing temperature below 45°C or above 55°C resulted in unspecific amplification products or in no amplification reaction (not shown). The amplification product obtained by the use of cDNA from OC 1, OC 2, OC 6 or bone marrow cells had a length of 534 bp which was in accordance with the expected size of the DNA encoding the whole mature part of HNL. Possible prepro sequences of the HNL at the 5'- and 3'-coding region could not be revealed by this approach but were not essential for cloning and expression of rHNL in *E. coli* [13].

Sequence determinations of the 534 bp OC 1, OC 6 and bone marrow cDNAs were carried out in the pCR II vector. Southern blot analyses were performed to obtain positive pCR II-HNL cDNA clones (Fig. 1B). This pCR II vector system takes advantage of the 3'-deoxyadenylate extension which the Taq DNA polymerases added to the termini of amplified cDNA [20] and allows direct cloning of the PCR product in appropriate constructed vectors (TA cloning). HNL oligonucleotide design therefore needed no additional unique restriction sites which were candidates for unspecific template hybridization yielding in various PCR amplification products.

The determined sequence data are shown in Fig. 2. The isolated 534 bp cDNA corresponded to the amino acid sequence of the mature HNL protein [2]. The HNL cDNA sequence using OC 6 transcripts as template is compared with the related lipocalin cDNA sequences of mouse (24p3) [4] and rat (α_2 microglobulin related protein) [3] indicating strong conserved nucleotide homologies with an overall conformity of

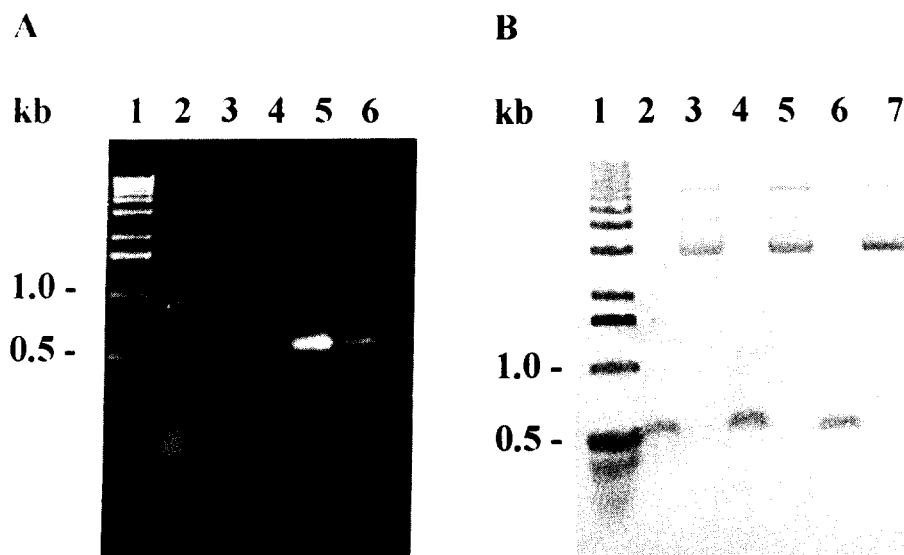


Fig. 1. (A) A 1% agarose gel electrophoresis of PCR products obtained by PCR reaction (see section 2) with inosine containing HNL primers and different templates. Lane 1, pBR 322 fragments (DNA Längenstandard X, Boehringer Mannheim, Germany) as a molecular weight standard. Template cDNA from; lane 2, OVCAR 3; lane 3, OC 1; lane 4, OC 2; lane 5, OC 6 and lane 6, bone marrow cells. The DNA samples were visualized by ethidium bromide fluorescence. (B) Southern blot analysis of *Eco*RI digested pCR II containing HNL cDNA. Lane 1, molecular weight standard. HNL cDNA from; lane 2, OC 1; lane 4, OC 6; lane 6, bone marrow cells. Lane 3, 5, 7 plasmids before digestion as control. Sequence analysis of the 534 bp cDNA revealed identity with the DNA backtranslate (Genetics Computer Group) of the mature HNL protein.

HNL	ctcttctctcc	tccggcacac	atcggaccta	gtagctgctg	aaaccatggg	50
rat			agaccta	gtagctgtgg	aaaccatggc	27
24p3						
HNL	cctgggtgtc	ctgtgtcttg	cccttgctct	gcttggggtc	ctgcagaggc	100
rat	cctgagtgtc	atgtgtcttg	gccttgccct	gcttggggtc	ctgcagagcc	77
24p3						
HNL	cagga	ctcGacgtcg	gacctgatcc	cgccccacc	tctgagcaag	45
rat	aggccc cagga	ctcaactcag	aacttgatcc	ctgccccacc	tctgatcagt	150
24p3	aggccc cagga	ctcaactcag	aacttgatcc	ctgccccacc	tctgctcact	127
HNL	gtccctctgc	agcagaactt	ccaggacaac	caattccagg	ggaagtggta	95
rat	gtccccctgc	agccaggett	ctggaccgaa	cggttccagg	gcaggtgggt	200
24p3	gtccccctgc	agccagactt	ccggagcgat	cagttccggg	gcaggtggta	177
HNL	tgtggtaggc	ctggcaggga	atgcaattct	cagag.aaga	caaagaccgc	144
rat	cgttgtcggc	ctggcagcga	atgcggtcca	gaaagaaaga	caaag..ccg	248
24p3	cgttgtgggc	ctggcaggca	atgcggtcca	gaaaaaaca	gaagg..cag	225
HNL	c.aaaagatg	tatgccacca	tctatgagct	gaaagaagac	aagagctaca	193
rat	ctttaccatg	tacagcacca	tctatgagct	acaggaagac	aatagctaca	298
24p3	ctttacgatg	tacagcacca	tctatgagct	acaagagaac	aatagctaca	275
HNL	atgtcacctc	cgctcctgtt	aggaaaaaga	a.....gtg	tgactactgg	237
rat	acgtcacctc	catcctcgtc	ag.....gg	gccagggctg	tcgctactgg	342
24p3	atgtcacctc	catcctggtc	agggaccagg	accagggctg	tcgctactgg	325
HNL	atcaggactt	ttgttccagg	ttgccagccc	ggcgagtcca	cgctgggcaa	287
rat	atcagaacat	tcgttccaag	ctccaggcct	ggccagtcca	ccctggggaa	392
24p3	atcagaacat	ttgttccaag	ctccagggtt	ggccagtcca	ctctgggaaa	375
HNL	cattaagagt	taccctggat	taacgagTta	cctcgtccga	gtgggtgagca	337
rat	tattcacagc	taccctcaga	tacagagcta	cgatgtgcaa	gtggccgaca	442
24p3	tatgcacagg	tatcctcagg	tacagagcta	caatgtgcaa	gtggccacca	425
HNL	ccaactacaa	ccagcatgct	atggtgtttc	tgaagaaagt	ttctcaaaac	387
rat	ctgactacga	ccagtttgcc	atggtatttt	tccagaagac	ctctgaaaac	492
24p3	cggaactaaa	ccagttcgcc	atggtatttt	tccgaaagac	ttctgaaaac	475
HNL	agggagtact	tcaagatcac	gctctacggg	agaaccaagg	agctgacttc	437
rat	aaacagtact	tcaaagtcac	cctgtacgga	agaaccaagg	ggctgtccga	542
24p3	aagcaatact	tcaaaattac	cctgtatgga	agaaccaagg	agctgtcccc	525
HNL	ggaactaaag	gagaacttca	tccgcttctc	caaattctctg	ggcctccctg	487
rat	tgaactgaag	gagcgattcg	tcagctttgc	caagtctctg	ggcctcaagg	592
24p3	tgaactgaag	gaacgtttca	cccgttttgc	caagtctctg	ggcctcaagg	575
HNL	aaaaccacat	cgtcttcccg	gtcccgatcg	atcaatggat	cgacggc	534
rat	ataaccaacat	cgtttttctct	gttccaccgc	accaatgcat	tgacaactga	642
24p3	acgacaacat	catcttctct	gtcccccacg	accaatgcat	tgacaactga	625
rat	acagacggtg	agcgtggctg	actgggatgt	gcagtggcct	gatggttcag	692
24p3	atgggtgggtg	agtgtggctg	actgggatgc	gcagagaccc	aatggttcag	675
rat	gtccacacgtg	tctgtctgcc	gctccatctt	tcctgttgcc	agagaatcac	742
24p3	gcgtgcctcg	tctgtctgcc	actccatctt	tcctgttgcc	agagagccac	725
rat	ctggctgccc	caccagccat	gattccatca	agcatctgat	ccc...tctt	789
24p3	ctggctgccc	caccagccac	cataccaagg	agcatctgga	gcctcttctt	775
rat	atttgatcag	ctctccccat	ccacctgtgt	taacgctgcc	ccaccaacgg	839
24p3	atttgccag	cactccccat	ccacctgtct	taa.....ca	ccaccaatg	819
rat	gctccccctt	tctgctgaat	aaacacatgt	ccccaaa		876
24p3	gcgtcccctt	tctgctgaat	aaatacatgc	cccc		853

Fig. 2. cDNA sequence of the mature HNL protein compared with the cDNA sequences of mouse oncogene protein 24p3 and rat α_2 microglobulin-related protein. Two clones of HNL cDNA derived from OC 6 (shown) and bone marrow cells show differences in four base pairs (big letters, see text). Boldface letters indicate base pairs conserved among the three lipocalins. Underlined letters indicate different base pairs compared with the cDNA sequence described by Bundgaard et al. [21].

71% (HNL, mouse), 72% (HNL, rat) and 86% (rat, mouse). Alignment of HNL-OC 6 cDNA with HNL-bone marrow cDNA shows base pair exchanges in four cases ($G^9 \rightarrow C$; $T^{315} \rightarrow C$; $G^{408} \rightarrow C$; $C^{507} \rightarrow T$) which, however, do not lead to an amino acid exchange. Bundgaard et al. [21] described

a cDNA of HNL from a chronic myelogenous leukemia cDNA library which differs at 10 highly degenerated codon positions (see Fig. 2) resulting also in the same derived amino acid sequence of rHNL protein. Further investigations are needed to show whether mutations of the cDNAs or/and the use of syn-

thesized primers for PCR [21] are responsible for the sequence differences found.

Computer HNL cDNA alignment of the three lipocalin motifs [25] with other lipocalin members (Fig. 3), like α_1 microglobulin [22], retinol binding protein [23] and α_1 acid glycoprotein [24] showed high homologies of 50–68%. In contrast to this there were only 30–40% homologies on the level of the amino acids. This underlined the conservation of these lipocalin motifs at the cDNA level and facilitates the screening for new members of the lipocalin family by PCR techniques using degenerated oligonucleotides designed according to the conserved sequence motifs.

In order to produce high amounts of the HNL protein for the purpose of functional and structural studies we expressed the protein in *E. coli*. For this purpose the pET 11 vector system [26] which allows the expression of the mature amino acid sequence starting with an initiating methionine was chosen. HNL cDNA containing a unique *Nde*I, a unique *Bam*HI site and a stop codon was selectively amplified with 'HNLR' and 'HNLF' oligonucleotides using pCR II-HNL plasmid as template. After restriction digestion the amplification product was ligated into the *Nde*I and *Bam*HI sites of the T7 expression vector 11a as previously described for TIMP-1 [13]. The plasmid pET 11a-HNL was transformed into *E. coli* strain HMS 174 [DE3]. In this host vector system the expression of rHNL was very efficient and rHNL was stored intracellularly in inclusion bodies in a denatured, insoluble form (not shown). Fifteen minutes after induction of the gene expression SDS-PAGE

1. motif

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HNL 61  aacttccaggacaaccaattccagggaagtggtatgtggtagcctggca
      N F Q D N Q E Q G K W Y V V G L A
rat 61  ggcttctggaccgaacggttcaggagcaggtggttcgttgcgacctggca
      G F W T E R F Q G R W F V V G L A
almg 43  aacttcaatattctctcggtatctatgggaagtggtacaacctggccatcgtt
      N F N I S R I Y G K W Y N L A I G
RBP 40  aacttcgacaaggctcgcttctctgggacctggtacgcatggccaagaag
      N F D K A R F S G T W Y A M A K K
alac 43  aacgccacctggaccagatcactggcaagtggtttatatcgcatcgcc
      N A T L D Q I T G K W F Y I A S A
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2. motif

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HNL 322  gtccgagtggtgagcacaactacaaccagcatgctatggtgttc
      V R V V S T N Y N Q H A M V F
rat 322  gtgcaagtggccgacactgactacgacctgttgcctatggtatlt
      V Q V A D T D Y D Q E A M V F
almg 301  tctctatgtggtccacaccaatgatgatgacatgttctctg
      S Y V V H T N Y D E Y A I F L
RBP 310  cactggatcgtcgacacagactacgacatgacgctacagtag
      H W I V D T D Y D T Y A V Q Y
alac 292  ttctgctcacttctgctgactcctcaggacaccaagacctacatgctt
      F A H L L I L R D T K T Y M L
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3. motif

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HNL 409  ctctacgggagaaacaaaggagctgacttcggaactaaaggagaacttc
      L Y G R T K E L T S E L K E N F
rat 409  ctgtacggaagaacaaaggagctgctcgatgaactgaaggagcatttc
      L Y G R T K G L S D E L K E R F
almg 391  ctctacgggagggcgccgagctgagggaaactctctgcaggacttc
      L Y G R A P Q L R E T L L Q D F
RBP 406  gtgttttccgggaccccaaggcctgccccagaagcgacagatt
      V F S R D P N G L P P E A Q K I
alac 376  gtctatgctgacaagccagagacgaccaaggagcaactggagagttc
      V Y A D K P E T T K E Q L G E F
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Fig. 3. Comparison of three conserved sequence motifs of some lipocalin family members [25]. DNA sequence numbers are related to the amino acid sequence of the mature proteins. Sequences were compared by the wordsearch program (Genetics Computer Group). Bold-face letters indicate the only invariant residue throughout the lipocalin superfamily tryptophan [7]. Footnotes: rat, rat α_2 microglobulin-related protein; almg, human α_1 microglobulin [22]; RBP, human retinol binding protein [23]; alac, human α_1 acid glycoprotein [24].

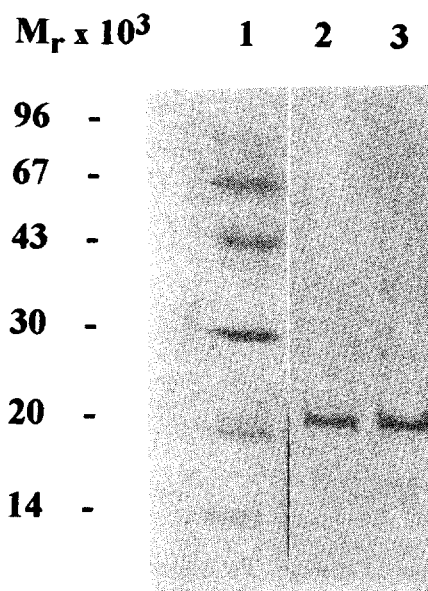


Fig. 4. Western blot analysis of *E. coli* cell pellet 4h after induction of rHNL gene expression with specific anti-HNL IgG [17] after SDS gelelectrophoresis: lane 1, molecular weight markers (Pharmacia, Freiburg, Germany); lane 2, rHNL (OC 6); lane 3, rHNL (bone marrow).

analysis indicated a production of an additional protein band of 21 kDa, the molecular weight of which was in accord with the calculated molecular mass of the HNL protein. Four hours after induction, the amount of the expression product was increased to about 15% of total bacterial protein. Western blot analysis of the *E. coli* cell pellet with rabbit polyclonal anti-HNL antibodies [17] showed cross reactivity with the recombinant 21 kDa protein indicating the production of rHNL (Fig. 4). The N-terminus was determined to be QDSTSDLPAPP which was identical to the one of the mature HNL. The starting methionine was processed by *E. coli* [27].

In this paper we demonstrated for the first time that ovarian cancer cells produce HNL mRNA. As neutrophils during leukodiapedesis, cancer cells have to penetrate through the basement membrane when they become invasive. Further investigations have to show whether the lipocalin contributes to this process.

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